Phospholipin, a novel heterodimeric phospholipase A2 from *Pandinus imperator* scorpion venom

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Abstract The primary structure of a phospholipase A2, with unique structural and functional characteristics, was determined. The large subunit has 108 amino acid residues, linked by a disulfide bridge to the small subunit, which contains 17 residues. Its gene was cloned from a cDNA library. The nucleotide sequence showed that the same RNA messenger encodes both subunits, separated only by a pentapeptide, that is processed during maturation.

Key words: Phospholipase A2; Amino acid sequence; Nucleotide sequence; Scorpion venom; *Pandinus imperator*

1. Introduction

Phospholipase A2 (PLA2) cleaves the ester bond at position 2 of the glycerol moiety of phospholipids, and is widely distributed in mammals, reptiles and arthropods [1]. Recently, the old classification of PLA2 into three groups [2] was expanded according to some structural and functional features, such as the number of disulfide bridges and the kind of amino acids responsible for the catalytic activity, found in the newly discovered PLA2 [3]. Dennis [3] reports seven well-defined PLA2 groups (I–VII) and adds three additional ones (VIII–IX), not fully characterized. Apart from the general hydrolytic function of these enzymes, several specific biological functions have been associated with members of the PLA2 superfamily. Among these functions are anti-inflammatory action [4], myonecrotic and muscle damaging effects [5] and ion channel blocking activity [6]. β-Bungarotoxin and crotoxin are two other well known toxic venom components with PLA2 activity [7,8]. In the venom of the scorpion *Pandinus imperator* an interesting heterodimeric phospholipase (IpTxi) was recently described. This protein causes inhibition of ryanodine binding to the Ca^{2+} channels present in skeletal muscle, very likely due to an indirect effect caused by the fatty acid liberated by the PLA2 activity of IpTxi [6].

In this communication we report the isolation and characterization of a novel heterodimeric protein from the venom of the same African scorpion *P. imperator*. The large subunit shares sequence similarities with the phospholipase moiety of IpTxi [6] and with those of phospholipases from honeybee and *Heloderma* lizard, but has very little or no effect on the inhibition of ryanodine binding to Ca^{2+} channels. The small subunit is unique to this heterodimeric phospholipase. We propose to call it phospholipin, and assume that together with IpTxi it will constitute a new group of PLA2, number X, following the classification proposed by Dennis [2].

2. Materials and methods

2.1. Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [6]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, dissolved in double-distilled water, centrifuged at 15 000×*g* for 15 min and the supernatant lyophilized. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved at least five fractions, of which number II contains phospholipase activity; this fraction was further separated into a carboxymethyl-cellulose (CM-cellulose) column, as described earlier for IpTxi [6]. One of the sub-fractions from CM-cellulose (number 3) contains phospholipin, which was finally obtained in homogeneous form by high performance liquid chromatography (HPLC), using a C18 reverse-phase column (Vydac, Hisperia, CA).

2.2. Phospholipase assay and determination of specificity

The egg yolk-agarose system of Habermann and Hardt [9] was used to follow the presence of phospholipase activity during the purification procedure. The enzyme specificity was determined using radioactively labeled substrates, and thin layer chromatography, as described previously [10].

2.3. Amino acid analysis and microsequencing

Amino acid analysis was performed in samples hydrolyzed in 6 N HCl with 0.5% phenol at 110°C in evacuated, sealed tubes as described [6]. Reduced and alkylated phospholipin, in amounts of 100 μg each time, was cleaved independently by three different enzymes, and the corresponding peptides were separated by HPLC, using the conditions described in the legend for Fig. 1. Digestion with *Staphylococcus aureus* protease V8 was performed in 100 mM ammonium bicarbonate buffer, pH 7.8, for 4 h, at 40°C, whereas hydrolysis with endopeptidases AspN and ArgC was performed in the conditions described elsewhere [11]. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [11].

2.4. Mass spectrometry determination

The molecular weight of pure phospholipin was determined by mass spectrometry, using a Kratos Kompact MALDI 3 v. 3.0.2 apparatus.

2.5. Cloning and sequencing

Two degenerated oligonucleotides encoding two different regions of phospholipin were synthesized, as previously described [6].

Oligo 1 (ATG TGG GAR TGY ACN AAR TGG TG-, where N is any nucleotide, R is A or G and Y is C or T-) corresponds to the DNA sequence of amino acids 2–10 of the large subunit, whereas oligo 2 (TGY GAR AAY GGN GTN GCN AC-) corresponds to the DNA sequence of amino acids 4–11 of the small subunit.

Total RNA was purified as reported [6]. Messenger RNA was purified following the instructions of the Hybond mAP protocol (messenger affinity paper; Amersham, RPN.1511). Synthesis of cDNA and the cloning of the cDNA library was performed as described [6,11]. The screening of the library was performed separately with oligonucleotides 1 and 2. The clone detected with oligonucleotide 1 was analyzed first. Inserts of cDNA from positive clones were amplified by polymerase chain reaction (PCR) using λ gt11 forward and reverse

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primers. PCR products were subcloned into pBluescript (pKS) phagemid. Clones of interest were sequenced using the Sequenase kit v. 2.0. (U.S. Biochemical Corp.). Oligos M13-20 and M13 reverse were used for sequencing [11].

3. Results and discussion

Fig. 1 shows the separation of soluble venom from *P. imperator*, by Sephadex G-50, CM-cellulose and HPLC. Fraction II (Fig. 1A) contains phospholipase activity. Sub-fraction 1 in Fig. 1B corresponds to IpTxi, capable of inhibiting ryanodine binding to skeletal muscle Ca^{2+} channels, as described by Zamudio et al. [6], whereas sub-fraction 3 (Fig. 1B) also contains a protein with phospholipase activity, which can be further purified by HPLC (inset Fig. 1B). The major component from the HPLC chromatogram (shown by the asterisk in the inset of Fig. 1) was assumed to be homogeneous, based on results obtained from SDS-gel electrophoresis (data not shown). It contains phospholipase activity but very little, if any, action on the inhibition of ryanodine binding to its receptor, contrary to IpTxi (data not shown). For this reason it was called phospholipin, without any additional reference to the ryanodine-sensitive Ca^{2+} channels. Mass spectrometry analysis showed that phospholipin was homogeneous, with a molecular mass of 14 841.2. Based on this analysis, the molecule was expected to contain in the order of 125 amino acid residues. When this protein was loaded into the microsequencer, two amino acid residues of about the same amount showed in each cycle, suggesting that it consisted of a heterodimeric protein. Reduction and alkylation of phospholipin permitted separation of the two subunits. This was obtained by gel filtration on a Bio-gel P-30 column, using the same system described for IpTxi [6]. Each one of the subunits was separately sequenced. Fig. 2A shows the results obtained. The short subunit was directly sequenced to the end, and contains only 17 amino acid residues, in which position 4 is occupied by a cysteine, responsible for the covalent attachment of the short peptide to the long one, through a disulfide bond. The larger subunit is composed of 108 amino acid residues and its complete sequence was determined after enzymatic cleavage. The sub-peptides were separated by HPLC (data not shown) and the overlapping sequences obtained are shown in Fig. 2A, underlining the overall amino acid sequence. Direct sequencing permitted the identification of the first 22 amino acid residues, whereas four sub-peptides obtained by cleaving with *S. aureus* protease V8 gave segments from amino acid residues in positions 20–37, 65–71, 73–88 and 101–108. The protease V8 cleavage was partial, since some of these fragments still contained internally situated glutamic acid residues, supposed to be cleaved by this enzyme, when the hydrolysis goes to completion. Additional positioning of residues was obtained by the use of two other proteinases. They correspond to the segment at positions 38–59 obtained by means of AspN protease hydrolysis, and sub-peptides 47–64 and 89–101, obtained by ArgC protease cleavage. It is worth mentioning that the specificity of ArgC was not 100% either; the batch of enzyme we used was able to cleave a lysine residue in position 46. The overall sequence determined by direct Edman degradation of the sub-peptides of phospholipin was additionally confirmed by the nucleotide sequence obtained from the cloned gene. The obtained cDNA nucleotide sequence of phospholipin is shown in Fig. 2B. The first gene cloned was performed using oligo 1 (see Section 2), designed to recognize the large subunit sequence, and the second was oligo 2. When the first cloned gene, based on the amino acid sequence of the large subunit (cloned with the help of oligo 1), was hybridized with oligo 2, specific for the short peptide, it was shown that both oligos were capable of recognizing the same clone. Under DNA sequencing analysis this initially misleading result was immediately understood. The gene that encodes both peptides is transcribed into the same mRNA, which encodes the large and the small subunit as well, separated by a pentapeptide Lys-Arg-Ser-Gly-Arg, which is processed during maturation, in quite the same way as initially demonstrated for the case of IpTxi, by our group [6]. The analysis and interpretation of Fig. 2B is still not complete, because there is a long 5′ non-translated segment of the cloned gene, whose function is still not certain. How-

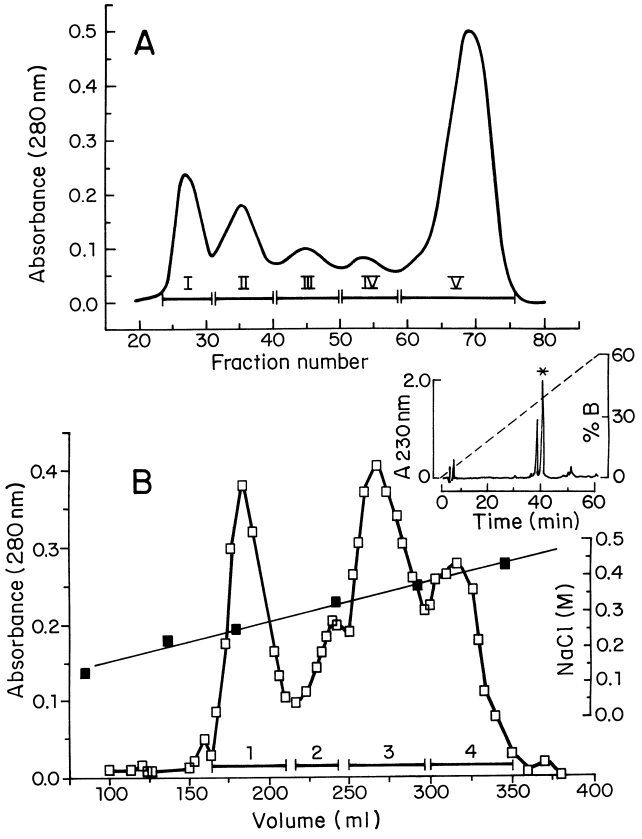

Fig. 1. Purification of phospholipin. A: Soluble venom from *P. imperator* (120 mg of protein) was applied to a Sephadex G-50 column (0.9×190 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7. Fractions of 5 ml each were collected and tested for their phospholipase activity. B: Tubes corresponding to fraction II were enzymatically active and were further applied to a CM-cellulose chromatographic column (0.9×30 cm) dissolved in the same buffer. The column was eluted with a linear gradient of sodium chloride, as indicated. Fractions 1 and 3 displayed phospholipase activity; the first contained IpTxi [6], whereas number 3 was finally separated by HPLC in a C18 reverse-phase column, as shown in the inset. Phospholipin corresponds to the major, last sub-fraction, labeled with an asterisk. A linear gradient was used from solvent A (0.12% trifluoroacetic acid in water) to 60% B (0.10% trifluoroacetic acid in acetonitrile). Phospholipin corresponds to approximately 2.4% of the soluble venom. The experiments aimed at determining its specificity, using radiolabeled phospholipids, clearly showed that phospholipin has phospholipase A2 type activity (results not shown).

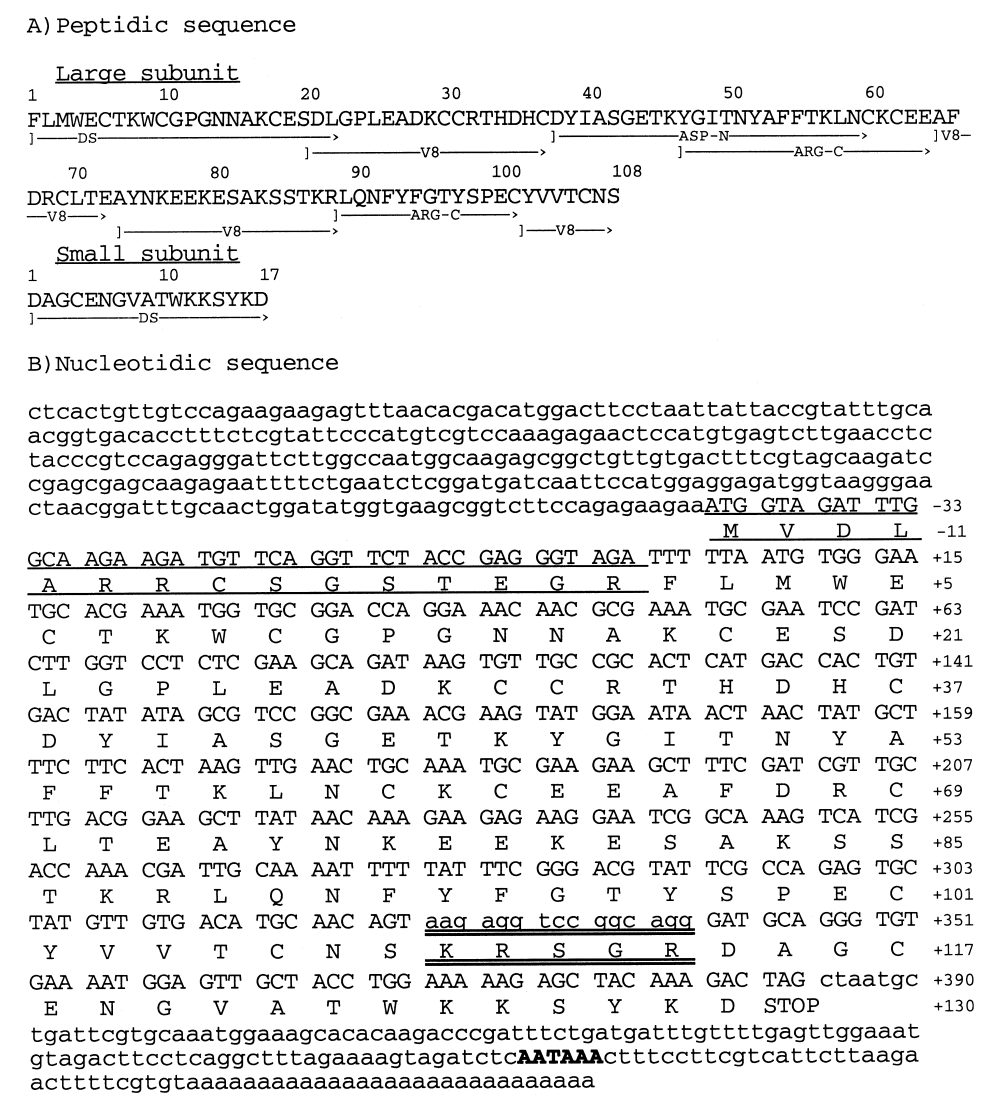

Fig. 2. Amino acid and nucleotide sequence of phospholipin. A: Primary structure of phospholipin as determined by direct sequencing of the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with protease V8 (labeled V8), endopeptidase AspN (labeled ASP-N) and endopeptidase ArgC (labeled ARG-C). B: Nucleotide sequence from the 5′ to the 3′ end of the clone that encodes phospholipin. The sequence corresponding to the putative signal is underlined, the pentapeptide eliminated during processing is double underlined, bold letters indicate the possible polyadenylation site, whereas lower case letters indicae the 5′ and 3′ non-coding regions of the gene. Numbers on the right hand side correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines).

ever, the sequence that encodes the heterodimer phospholipin molecule is very clear. It is composed of 393 nucleotides (including the stop codon), starting at residue 1 with Phe (see line 8 in Fig. 2B) labeled on the right hand side with number +5 (which corresponds to glutamic acid), and ends at residue 130, just before the stop codon. In this sequence there is a pentapeptide (double underlined) that is processed during maturation, because it was not seen when we sequenced the heterodimeric peptides. There is a putative signal peptide (single underlined in positions −15 to 0), but there are still 304 nucleotides to the left side, with unknown function. That is, the long 5′ non-translated sequence (lower case letters) could contain information for a longer signal peptide, that might start at the ATG codon situated 9 residues to the left of the first Met residue (our amino acid −15 in Fig. 2B) implying that the signal peptide could start at amino acid −24. It is also not clear if the putative signal peptide we have labeled is, in fact, the signal peptide. We are concerned about the presence of two Arg residues within this segment. Thus, the possibility that this segment corresponds to the message for translating an unknown third peptide or long propeptide needs further analysis and work. Also, the long 3′ non-translated region (104 nucleotides), before the putative polyadenylation site (bold capital letters at the end of the figure) needs some additional work.

Although the primary structure of the large subunit of phospholipin presents a close similarity with PLA2 from honey bee and *Heloderma* lizard, which would place it in the group III phospholipases, it is not certain that it fits there. One of the main reasons is because it is a heterodimer in which the small subunit (17 amino acid residues) has no resemblance to any other protein known. It seems clear that the large subunit moiety is the one bearing the phospholipase activity, in all three examples mentioned. Also β-bungarotox-

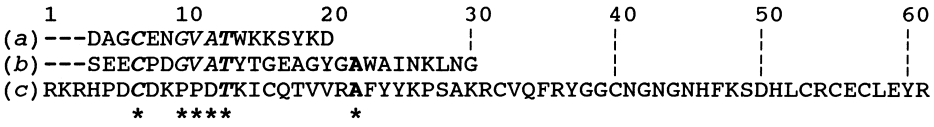

Fig. 3. Comparative analysis of the small phospholipase subunits. The amino acid sequence of the small subunits of phospholipin (this work), and those of IpTxi [6] and β2-bungarotoxin [13] were compared, after alignment of the first cysteinyl residues by adding gaps (-) to the sequences of phospholipin and IpTxi. Very little identity exists. Phospholipin (a) and IpTxi (b) share five identical amino acid residues at the same position (18% identity), whereas with the small β1 subunit of bungarotoxin (c) only two positions were occupied by identical residues (less than 4% identity). Bold italics indicate identical residues in all three sequences, italics indicate identity for only two of the sequences on pairwise comparison. Identical residues are highlighted by asterisks.

in, a presynaptically active phospholipase isolated from the snake *Bungarus multicinctus* [7], and IpTxi, isolated from the scorpion *P. imperator* [6] are heterodimers, but again there is no resemblance in structure or function, when we compare the small subunits of these three venom components, as shown in Fig. 3. Also functionally, the short peptide of β-bungarotoxin with 61 amino acid residues was suggested to be a K^+ channel blocker [12], whereas that of IpTxi (27 amino acid residues) apparently has no measurable function towards Ca*2*+ channels or towards any other of several ion channels assayed (see [6]). Phospholipin has the shortest of all the small subunit peptides of the three heterodimeric phospholipase known, and we do not know either what its function is. The three-dimensional structure of phospholipin was modelled using the Swissmod program and facilities (data not shown), and the results of the model suggests that cysteine at position 4 of this 17 amino acid peptide is bound to cysteine in position 101 of the phospholipase moiety. Structurally, phospholipin shows two additional cysteines (one more disulfide bridge), not present in IpTxi, which might be responsible for folding phospholipin in a different manner than that of IpTxi. It can be speculated that this structural difference is responsible for a distinct substrate specificity, hence a different biological action, such as the indirect effect on the binding of ryanodine to the Ca^{2+} channels, described for IpTxi. Due to these characteristics, phospholipin described here, although similar to group III phospholipases A2, seems to constitute the prototype of a novel group of PLA2, which would be number X, according to the classification recently proposed by Dennis [3].

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